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13. ABSTRACT ***(Maximum 200 Words)***

The object of this project is to develop a high-throughput method for screening potential inhibitors of breast cancer cell haptotaxis and chemotaxis, and to apply this method to identify signaling events mediating constitutive migration of malignant breast cells. The pathways that control these signaling events may be targets for development of new classes of anti-tumor drugs. The significant advances made during the first year of this project include: design and manufacture of the hardware necessary to develop the high-throughput screen, development of an efficient, high-throughput migration assay compatible with drug screening, and formation of a collaboration with the Developmental Therapeutics Program to screen their plated and natural products repositories for new inhibitors of breast cancer cell migration. Our mechanistic work has focused on construction of cell lines overexpressing normal and mutant forms of candidate signaling molecules involved in breast cell migration, including G protein alpha subunits, focal adhesion kinase (FAK), and crk associated substrate (CAS). Our work in the second year will focus on screening compounds and characterizing these cell lines to find new inhibitors of migration and new targets for drug design, respectively.

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Introduction

The purpose of this project is to identify the mechanisms governing the constitutive migration of breast cancer cells, with an eye towards reducing the severity of malignant breast cancer by inhibiting the signaling pathways responsible for maintaining this migration. The approaches proposed in this project focus on developing an in vitro migration assay suitable for identifying new biochemical inhibitors of tumor cell migration, and on isolating a potentially novel integrin receptor that mediates constitutive migration of breast cancer cells on the extracellular matrix protein laminin-5.

Body

The goals of this project have been defined in two specific aims. Progress has been made in each aim, according to the schedule outlined in the Statement of Work. The accomplishments for each Aim are described below.

Specific Aim 1: Define the components of the pertussis toxin-sensitive G protein signalling pathway that mediates migration of normal and malignant breast cells on laminin-5.

Specific elements of this aim that were to be addressed in the first 12 months of this project are:

Task 1. Optimize assays for identifying signalling proteins in migrating cells, Months 1-6.

In addition to the work directed at our specific tasks (described below), we expanded our signaling assays to include Ca^{+2} fluxes in response to binding extracellular matrix proteins. This is in conjunction with our drive to develop a fluorescence based cell migration assay, and it will allow us to plot changes in Ca^{+2} levels over time in migrating and inhibited populations in breast cells. This project was spurred by our observation that calcein, a fluorescent dye that binds Ca^{+2} and is commonly used to tag living cells, inhibits migration of our cells on laminin-5. While this interferes with our efforts to perform kinetic measurements of cell migration, it led us to investigate the importance of Ca^{+2} in controlling migration. We have since discovered that Ca^{+2} levels are directly proportional to migration rate in our cells, and that both of these factors vary considerably on different extracellular matrix proteins. We would therefore like to pursue this in the next three years of this grant.

a. Develop protein kinase and lipid kinase assays using whole cell lysates as starting material

Progress: We decided after performing initial assays that we would look for specific protein activities rather than take the longer process of deducing activities from whole cell lysates. Towards this end, we formed a collaboration with Dr. Tim O'Toole at The Scripps Research Institute to investigate the function of receptor for activated c-kinase-1 (RACK1) in controlling the migration of breast cells. This collaboration was spurred by Dr. O'Toole's discovery that RACK1 binds to the cytoplasmic tail of the β1 integrin chain. We contacted Dr. O'Toole after he made this discovery using a yeast two-hybrid screen. Because he had no immediate functional assays to test the significance of this association, we took it upon ourselves to examine the consequences of RACK1 overexpression in breast cancer cells. Because RACK1 associates with activated forms of protein kinase C, we hypothesized that overexpression of RACK1 would stimulate migration in our cells. Initial results from stably transfected MCF-7 cells supported this hypothesis, but these cells proved difficult to maintain in culture. We subsequently learned that overexpression of RACK1 in established cells inhibits the cell cycle in fibroblasts, and upon re-examining our transfected lines discovered that while they continued to grow in G418 selection medium, they produced no

exogenous RACK1. To address the problem of long-term downregulation of RACK1 expression in our cells, we switched to transient transfections but were unable to observe any changes in cell migration of transiently transfected cells. Finally, we examined the migration of fibroblasts stably transfected with RACK1 and discovered a consistent reduction in migration on many extracellular matrix substrates. This proved to be true in control transfectants lacking the RACK1 gene as well, such that we were unable to ascribe any specific function to RACK1 overexpression in any of the cells we have tested. The project was abandoned after a full year of effort, due to lack of a coherent story.

We also examined the role of the small heat shock protein HSP27 in controlling migration of breast cancer cells. Using MDA-MB-231 cells stably transfected with HSP27 (provided by Dr. Steven Carper, UNLV Department of Chemistry), we determined that while overexpression of HSP27 does not influence haptotactic migration of these cells on laminin-5, HSP27 does render these cells resistant to inhibitors of signaling molecules required for cell migration. Also, factors that enhance phosphorylation of HSP27 enhance migration in these cells. This work was published in Molecular Cell Biology Research Communications. Because we did not get clear cut inhibition or stimulation of migration by HSP27 expression alone, and because we were able to preserve consistent levels of HSP27 overexpression in our cells, we deemed this project too risky to continue and thus terminated it after acceptance of our manuscript.

Continuing with our philosophy that examination of specific signaling proteins is better than searching through whole cell lysates, we turned our attention to two other known signaling proteins known to be linked to integrin receptors, focal adhesion kinase (FAK) and crk associated substrate (CAS). We formed collaborations with Dr. Thomas Parsons and Dr. Amy Bouton at the University of Virginia School of Medicine, because they are experts in the function of these proteins. The hypothesis we wish to test is that FAK and CAS are required for haptotactic migration of normal and malignant breast cells on laminin-5, and that FAK/CAS signaling pathways are constitutively active in malignant cells. Drs. Parsons and Bouton have supplied us with cDNA constructs of wildtype and mutant forms of FAK and CAS, and at the end of this first year of the grant we have finished subcloning these constructs in mammalian expression vectors in preparation for transfections in the second year.

We also obtained cDNA constructs for a variety of pertussis toxin-sensitive G protein subunits from Drs. Randy Reed (Johns Hopkins University), Bradley Denker (Harvard University) and Louis Ercolani (Harvard University). We also have pertussis toxin-insensitive mutants of Gαi1, 2, and 3. These too have been subcloned into mammalian expression vectors, and at the end of this first year we have transfected two constructs, containing wildtype and GTPase deficient Gαo, into MCF-7 cells. The hypothesis we wish to test with these cells is that overexpression of constitutively active subunits will enhance cell migration and that this migration will not be inhibited by pertussis toxin, while wildtype constructs will be pertussis toxin sensitive.

b. Optimize methods for isolating integrin complexes

Progress: We capitalized on our observation that integrins can be purified from cells plated on laminin-5 by extracting the cells with NH_4OH to generate integrin-laminin-5 complexes in situ. Our hypothesis was that integrins purified from cells in a "functional" state (i.e., still attached to their ECM ligand) would provide the best affinity matrix for binding soluble signaling molecules. Further, we believed that integrin complexes isolated from migratory and non-migratory populations of cells might adopt distinct conformations and thus bind distinct populations of signaling molecules. We

tested these hypothesis by purifying integrin receptors from MCF-7 and MCF-10A cells and incubating them with ^{35}S methionine and $^{32}P_i$ radiolabeled detergent extracts taken from separate populations of these cells. We failed to observe any specific binding of proteins in these extracts to plates containing integrin receptors when compared to control plates coated with nonfat dried milk or bovine serum albumin. The project was abandoned after six months.

c. Optimize sensitivity and throughput of non-fluorescence, dye-based cell migration assay.

Progress: We obtained a grant from the UNLV Office of Sponsored Programs to pursue a collaboration with Polyfiltronics/Whatman, Inc. to design and manufacture a 96 well, fluorescence-based migration filter plate. During the first year of this grant we tested prototypes of the plates and recommended changes to the design. At the close of the first year, we were entangled in some legal disputes with Polyfiltronics concerning their failure to made changes to the plate design that we requested. It is hoped that these issues will be resolved early in the second year so that full scale use of the plates can begin. With the few plates we have obtained, we have developed a fully functional assay that is capable of measuring the migration of a single population of cells over time and that can also determine the viability of these cells at the end point. This means we can distinguish cytotoxic and non-cytotoxic inhibitors of cell migration with exquisite sensitivity and high throughput. As a result, we have received permission (signed Material Transfer Agreements) from the Developmental Therapeutics Program at the National Cancer Institute to screen their plated compounds and natural product extracts repositories for non-cytotoxic inhibitors of breast cancer cell migration. I would rank this as our most significant achievement of the first year in this grant.

Task 2. Identify integrin-associated signalling proteins in breast cells migrating on laminin-5, Months 6-30.

a. Apply signalling assays to isolated integrin complexes from migrating and non-migrating cell populations.

Because we have thus far been unable to purify integrin complexes with any specificity, we have not made any progress in this aim. It is our intention to apply signaling assays to immunoprepitated integrin complexes from cells transfected with specific cDNAs described above. This will happen in the second year of the grant.

b. Identify pertussis toxin- and cholera toxin-sensitive proteins in migrating cell populations.

See Progress on Specific Aim 2, below.

c. Screen active signalling molecules in integrin complexes for known proteins using existing antibodies by western blot and immunoprecipitation.

Thus far this has not been necessary since we are targeting defined proteins via transfection studies. We are able to measure activity/expression levels of a variety of signaling molecules in our cells, including adenylate cyclase, FAK, MAP kinase, and src.

Task 3. Identify inhibitory compounds that block migration of malignant breast cells on laminin-5, Months 12-30.

a. Optimize sensitivity and throughput of fluorescence-based cell migration assay.

We have already begun work on this aim, and thus are somewhat ahead of schedule. We screened a few known anti-cancer drugs during the development phase of our in vitro migration assay, and found that perillyl alcohol, a derivative of D-limonene, inhibits migration at sub-cytotoxic doses and thus it is being used as a model target for optimization of our screening method. Likewise, we have discovered that the calcium channel inhibitor carboxyamido-triazole (CAI, provided by Dr. Elise Kohn at NCI) inhibits migration at sub-lethal doses, and this is also being used to optimize sensitivity of our assays. Our current protocol has been optimized for DMSO toxicity for four cell lines (MCF-10A, MCF-7, MDA-MB-231, MDA-MB-435) in anticipation of screening the DTP plated compounds, all of which are solubilized in DMSO.

b. Screen inhibitors of biochemical signalling pathways in migration assays.

Once we get the legal troubles with Polyfiltronics solved, we expect to receive large quantities of our migration plate, and will begin screening the DTP compounds in earnest.

Specific Aim 2: Identify the specific protein targets of compounds that inhibit migration of normal and malignant breast cells on laminin-5.

Progress: We had previously observed that pertussis toxin inhibits the haptotactic migration of immortalized and malignant breast cells on laminin-5. To identify the target for this compound, we performed ADP ribosylation assays with radiolabeled substrates, followed by immunoprecipitation with antibodies specific for Gαi1, 2, and 3. We found that pertussis toxin targets Gai3 in immortalized (MCF-10A) and weakly malignant (MCF-7) cells but targets Gαi1 in highly malignant (MDA-MB-231 and MDA-MB-435) cells. This led us to request the cDNA constructs for these proteins as described above.

Our future plans include characterizing the target of perillyl alcohol that is required for migration in our cells. This will take place in the second year of the grant.

Appendices

key research accomplishments

- Design and manufacture of 96-well fluorescence-based cell migration plates
- Establishment of automated cell migration/cytotoxicity assay suitable for screening thousands of potential inhibitors of breast cancer cell migration per year
- Establishment of cooperative agreement with Developmental Therapeutics Program at NCI to screen plated compounds and natural products repositories for inhibitors of breast cancer cell migration
- Establishment of collaboration with Drs. Tom Parsons, Amy Bouton, and Louis Ercolani to determine the function of FAK, CAS, and Gai proteins in controlling migration of breast cells on laminin-5
- Established collaboration with Dr. Dan Kirgan, University of Nevada School of Medicine, to study the effects of migration inhibiting drugs on chemotaxis and haptotaxis of primary tumor cells derived from surgically removed breast tumors
- Eastablished research methods for examining Ca^{+2} signaling in migrating populations of breast cells

Reportable Outcomes:

manuscripts

Rust, W., K. Kingsley, T. Petnicki, S. Padmanabhan, S.W. Carper, and G.E. Plopper. Hsp27 plays two distinct roles in controlling human breast cancer cell migration on laminin-5. Molecular Cell Biology Research Communications, 1:196-202, 1999.

abstracts

Rust, W.L., T. Petnicki, S. Carper, and G.E. Plopper. HSP27 enhances MAP kinase dependent migration of breast cancer cells. Mol. Biol Cell 9:288a, 1998.

Rust, W.L., J.L. Huff, and G.E. Plopper. A high throughput assay for screening anti-migratory compounds. Submitted for 1999 Annual Meeting of American Society for Cell Biology.

Carroll, K., Stimulation of breast cell migration on laminin-5 by antibody induced activation of $\alpha 3\beta 1$ integrin receptor and a $G\alpha i3$-mediated signaling pathway. Submitted for 1999 Annual Meeting of American Society for Cell Biology.

presentations

University of Nevada School of Medicine, Department of Surgery, Grand Rounds. June 24th, 1999. Title: In Vitro Analysis of Cancer Cell Migration.

patents and licenses applied for and/or issued

N/A

degrees obtained that are supported by this award

N/A

development of cell lines, tissue or serum repositories

N/A

informatics such as databases and animal models, etc.

Development of novel automated cell migration/cytotoxicity assay

funding applied for based on work supported by this award

1. UNLV student research on HSP27 and breast cancer. $5,000. Submitted 9/20/98 to UNLV Alumni Association.
2. UNLV student research on effects of electromagnetism on breast cancer. $5,000. Submitted 9/20/98 to UNLV Alumni Association.
3. Integrin signaling and migration of breast cancer cells on laminin-5. American Cancer Society research project grant, $444,000. Start date June 1, 1999, end May 31, 2002. Submitted October 15, 1998.
4. VEGF induced hyperpermeability and its role in tumor metastasis in intact microvessels. Whitaker Foundation Biomedical Engineering Research Grant preproposal (Bingmei Fu, PI; George Plopper, Co-Investigator). Submitted December1, 1998. No start/stop dates indicated. Invited to submit full application as of December 22, 1998.
5. Cell adhesion proteins, electromagnetic fields, and breast cancer. Western Alliance for Expanding Student Opportunities (Arizona State University) $1,378. Spring 1999 Semester. Submitted December 3, 1998.
6. Electromagnetic field modification of cell adhesion proteins. Western Alliance for Expanding Student Opportunities (Arizona State University) $1,378. Spring 1999 Semester. Submitted December 3, 1998.
7. UNLV Site Grant, $5,000, submitted December 28, 1998. Proposed dates: April 1, 1999-December 31, 1999.
8. Biomedical Engineering and Anti-cancer Drug Design. Strategic Planning Initiative Grant, $30,000. Bingmei Fu, Ph.D., Co-investigator. Submitted December 28, 1998. Start date: June 1, 1999. Stop date: May 31, 2000.
9. UNLV Strategic Planning Initiative Grant. Title: Biomedical engineering: Blood flow and cancer therapy. Total amount: $30,000. July 1, 1999-June 30, 2000. Submitted January 22, 1999.
10. UNLV Strategic Planning Initiative Grant. Title: Establishment of the UNLV Cancer Institute (Officially listed as collaborator, Steven Carper is PI). Total amount: $30,000. July 1, 1999-June 30, 2000. Submitted January 22, 1999.
11. United States Army Breast Cancer Research Program, Idea Grant. Title: Application of a high-throughput in vitro assay to identify inhibitors of human breast cancer cell migration. Total amount, $335,250. June 1, 2000-May 31, 2003. Submitted June 2, 1999.
12. United States Army Breast Cancer Research Program, Predoctoral Training Grant. Title: Elucidation of pertussis toxin-sensitive migration signaling in human breast cancer cells (Officially listed as mentor: Will Rust is PI). Total amount, $66,000. June 1, 2000-May 31, 2003. Submitted June 2, 1999.
13. United States Army Breast Cancer Research Program, Postdoctoral Training Grant. Title: The Role of Focal Adhesion Kinase and CAS in Integrin-mediated Signaling on Distinct Forms of Laminin-5

(Officially listed as mentor: Janice Huff is PI). Total amount: $143,889. June 1, 2000-May 31, 2003. Submitted June 2, 1999.

14. WAESO fall 1999 minority student research project. Title: Signal Transduction in Human Breast Cancer Cells. Total amount, $1,378. Submitted May 1, 1999.
15. The Whitaker Foundation, Biomedical Engineering Research Grant (Officially listed as collaborator: Bingmei Fu, UNLV Dept. of Mechanical Engineering, is PI). Title: VEGF induced hyperpermeability and its role in tumor metastasis in intact microvessels. Total amount: $205,675. December 1, 1999-November 31, 2002. Submitted June 1, 1999.
16. American Institute for Cancer Research, Investigator Initiated Grant. Title: Application of an automated assay to discover natural inhibitors of cancer cell migration. Total amount, $149,923. Submitted July 1, 1999.
17. UNLV Applied Research Program. Title: A UNLV/Biotechnology Collaboration. Total amount, $577,816. September 1, 1999-August 31, 2001. Submitted August 5, 1999.

employment or research opportunities applied for and/or received based on experiences/training supported by this award

N/A

a copy of each of the above cited manuscripts and abstracts.

See attached.

Abstract

American Society for Cell Biology 39th Annual Meeting
Submitted by William Rust, University of Nevada, Las Vegas

A High Throughput Assay for Screening Anti-Migratory Compounds

Large scale screening strategies aimed at finding anti cancer drugs traditionally focus on identifying cytotoxic compounds that attack actively dividing cells. Because progression to malignancy involves acquisition of an aggressively invasive phenotype in addition to hyperproliferation, simple and effective screening strategies for finding compounds that target the invasive aspects of cancer progression may prove valuable for identifying alternative and preventative cancer therapies. Here we describe a kinetic, fluorescence based automated assay for identifying anti-migratory compounds, with the ability to discern cytotoxic from non-cytotoxic modes of action. With this assay, we show that carboxy-amido triazole (CAI) effectively inhibits migration of two breast cancer cell lines (MCF-10A, MDA-MB-435), while leaving the cells viable, whereas the common breast cancer treatment drug tamoxifen arrests migration and leads to cell death. This technology lends itself to high-throughput screening and may be a valuable too for future drug discovery.

William L. Rust, Janice L. Huff, George Plopper
Department of Biological Sciences
University of Nevada, Las Vegas

Stimulation of breast cell migration on laminin-5 by antibody induced activation of α3β1 integrin receptor and a Gαi3-mediated signaling pathway

Kilpatrick J. Carroll[1], Vincent J. Cavaretta[1], Russell W. Bandle[1], Janice L. Huff[1], Martin Alexander Schwartz[2], Vito Quaranta[2], George E. Plopper[1], [1] University of Nevada Las Vegas, 4505 Maryland Parkway, Las Vegas, NV 89154-4004, [2] The Scripps Research Institute

We have recently shown that malignant breast cells constitutively migrate on laminin-5, a component of breast basement membrane, while normal and immortalized (non-malignant) breast cells do not. In this study we focused on identifying signaling pathways that control migration in these cells. We report that direct stimulation of the α3β1 integrin receptor with the β1 stimulating antibody TS2/16 was sufficient to induce haptotactic migration of the immortalized, non-malignant breast cell line MCF-10A on laminin-5, but not on laminin-1. TS2/16-stimulation also induced a rapid rise in intracellular cyclic AMP within 20 minutes after these cells were plated on laminin-5. Pertussis toxin (PTX) inhibited both haptotactic migration and the rise in cyclic AMP levels in TS2/16-stimulated cells. The Gαi3 subunit of heterotrimeric G proteins was identified as the target of PTX in immunoprecipitates of ADP-ribosylated proteins following PTX exposure. TS2/16-stimulated migration on laminin-5 was also blocked by inhibitors of adenylate cyclase (SQ22536) and protein kinase A (H-89), suggesting that these signaling proteins may form an integrin-associated, Gαi3-linked signaling pathway in MCF-10A cells.

Molecular Cell Biology Research Communications 1, 196–202 (1999)
Article ID mcbr.1999.0130, available online at http://www.idealibrary.com on IDEAL®

Heat Shock Protein 27 Plays Two Distinct Roles in Controlling Human Breast Cancer Cell Migration on Laminin-5

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Received May 20, 1999

It has recently been reported that phosphorylation of the small heat shock protein 27 (hsp27) enhances p38 MAP kinase dependent migration of bovine and human vascular endothelial cells. We have examined the role of hsp27 in controlling the constitutive migration of human breast cancer cells on the extracellular matrix molecule laminin-5. In a haptotaxis assay, anisomycin- or heat shock-induced phosphorylation of hsp27 enhances migration of MDA-MB-231 breast cancer cells constitutively overexpressing hsp27. Under these conditions, hsp27 redistributes to the nucleus. Unphosphorylated hsp27, which remains in the cytosol, induces resistance to a subset of drugs that inhibit haptotactic migration of these cells. We conclude that hsp27 plays two distinct roles in controlling migration of breast cancer cells: phosphorylated hsp27 enhances migration, while unphosphorylated hsp27 can sustain migration in the presence of inhibitory drugs. ©1999 Academic Press

Key Words: heat shock protein 27; migration.

Heat shock protein 27 (hsp27) is a member of the heat shock family of proteins, which confer resistance to a variety of cellular stresses (reviewed in 1, 2). Hsp27 over-expression confers resistance to acute heat shock and some anti-cancer drugs (1, 3). hsp27 function is thought to be controlled by its phosphorylation state and intracellular localization, however, the exact mechanism remains unclear.

Following heat shock, hsp27 is phosphorylated and redistributes to the nucleus (1). This phosphorylation is mediated by mitogen activated protein kinase activated protein kinase 2/3 (MAPKAP K2/3) (1), which in turn is activated by upstream kinases ERK 1/2, JNK/SAPK, and p38 MAPK (2–4). Phosphorylation-deficient mutants do not confer heat shock resistance, suggesting that phosphorylation is critical to its ability to protect cells from heat (3).

In vitro, hsp27 inhibits actin polymerization (2, 3). *In vivo,* hsp27 complexes are sometimes associated with actin filaments in motile cell protrusions such as lamellipodia, filopodia, and membrane ruffles (5), suggesting it may play a role in controlling cell motility or cytokinesis, though its exact role in these activities is unclear (6, 7).

Extracellular matrix (ECM) proteins play a critical role in controlling numerous functions in virtually all cells, including migration (8, 9). The ECM protein laminin-5 is abundantly expressed in the basal lamina of most epithelial tissues, where it promotes growth, differentiation, and migration of epithelial cells (10–12). In the breast, laminin-5 is the preferred adhesive substrate for normal breast epithelial cells and mediates constitutive migration of breast cancer cells (9). The relationship between hsp27 over-expression and laminin-5 mediated migration in breast cells has thus far been unexplored.

In the present study, we show that hsp27 overexpression alone does not affect constitutive migration of MDA-MB-231 human breast cancer cells towards laminin-5. However, phosphorylation of hsp27 by heat shock or MAPK activation enhances this migration over control cells, concomitant with its re-localization to the nucleus. In addition, unphosphorylated hsp27 confers resistance to drugs that inhibit cell migration, and is localized to the cytosol. From these results, we conclude that hsp27 modulates migration on laminin-5 by two distinct mechanisms, that can be distinguished by hsp27 phosphorylation state: phosphorylated hsp27 enhances migration, while unphosphorylated hsp27 offers protection against migration inhibitors. These two distinct functions may reflect the dual role of hsp27 as

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an actin polymerization modulator and molecular chaperone, respectively.

MATERIALS AND METHODS

Cells. MDA-MB-231 cells were maintained as previously described (9). DB46 cell line was made by transfecting MDA-MB-231 cells with an hsp27 constitutive expression vector (pβ27), constructed by cloning the human full length hsp27 cDNA (13) fragment into the pHβAPr-1 neo (14) under the control of the β actin promoter as previously described (15). Control cell line DC4 was made by transfecting cells with the same plasmid lacking the hsp27 cDNA sequence.

Materials. SQ22536, KT5823, H89, Bisindolylmaleimide, Genistein, and Anisomycin were purchased from Calbiochem (San Diego, CA). PD98059 was purchased from New England Biolabs (Beverly, MA); and pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Purified laminin-5 was generously provided by Desmos, Inc. (San Diego, CA).

Immunoblotting. Cells were scraped and lysed in RIPA lysis buffer, and lysates subjected to western blot as previously described (16). In this instance, primary antibodies were anti-hsp-27 monoclonal antibody (1:1000 dilution), Stressgen (Victoria BC, Canada) or anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody (1:2500 dilution), New England BioLabs (Beverly, MA), and alkaline phosphatase conjugated anti-mouse monoclonal antibody was used as the secondary antibody.

Quantification of apoptosis. Apoptosis was quantified using an ELISA assay kit (Boehringer Mannheim, Indianapolis, IN). Briefly, DC4 and DB46 cells were treated with indicated concentrations of sodium butyrate for 20 hours, then solubilized according to manufacturers protocol. ELISA wells were coated with anti-histone antibody, then loaded with cytoplasmic extracts, and finally, incubated with anti-DNA secondary antibody conjugated with peroxidase. Absorbance of peroxidase substrate was measured at 405nm using a Microplate autoreader (Dynatech MR5000).

Heat shock survival. Survival after exposure to 45°C for various time intervals was determined by colony forming assay as previously described (17). Briefly, cells were heat shocked at 45°C, then allowed to grow for 9–12 days. Colonies (>50 cells) were stained with crystal violet and counted. Percent survival is expressed relative to control, unshocked cells.

Transwell haptotactic migration assay. Cell migration was determined as previously described (9) except that cells were stained with 5 μM calcein AM, Molecular Probes (Eugene, OR) added directly to the migration wells 30 minutes prior to measuring migration. To quantitate migration, the top side of each filter was wiped with a cotton tipped applicator to remove cells that had not migrated through the filter, and fluorescence of the incorporated dye was measured from the filter with a fluorescence plate reader. Relative fluorescence values for each experimental condition are expressed relative to control, untreated samples.

Phosphorylation assay. Cells were pre-incubated with 100 μCi/ml ^{32}P-orthophosphate, NEN (Boston, MA), in 90% phosphate-free medium for four hours, then subjected to either heat or drug treatment for 30 minutes. Cells were lysed in RIPA, and 15 μg of cell lysates was immunoprecipitated with anti-hsp27 antibody and protein A/G agarose, Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitates were separated by SDS-PAGE (12% acrylamide), and the dried gel was exposed to film. MAP kinase phosphorylation was determined by immunoblotting with monoclonal antibody specific for phosphorylated MAP kinase.

Miscellaneous. Cell adhesion, immunoprecipitation, and indirect immunofluorescence assays were performed as previously described (16), using anti-hsp-27 monoclonal antibody and anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) monoclonal antibody (New England BioLabs, Beverly, MA) as primary antibodies where indicated. For affinity captured laminin-5 assays, untreated wells were coated with anti laminin-5 monoclonal antibody TR-1 (20 ug.ml) in 100 mM carbonate buffer (pH 9.3) for one hour at room temperature. After washing and blocking with blotto, wells were incubated for 1 hour at room temperature with 804G cell conditioned medium, thereby allowing for "capture" of soluble laminin-5. Wells were then washed twice with PBST.

RESULTS

The Human Breast Cell Line DB46 Stably Overexpresses hsp27

To examine the effect of hsp27 over-expression on the behavior of human breast cancer cells, we stably transfected clones of MDA-MB-231 cells with the pβ27 plasmid containing full length human hsp27 cDNA under the control of the β-actin promoter (15). Overexpression of hsp27 in transfected clones was confirmed by Western blot (Fig. 1A), and function of hsp27 in these cells was determined by resistance to sodium butyrate-induced apoptosis and heat shock (Fig. 1B, C). We found that DB46 cells expressed 2-3 fold more hsp27 and exhibited increased (10- to 100-fold greater) survival when compared to control DC4 cells. From these results we conclude that DB46 cells constitutively over-express functional hsp27.

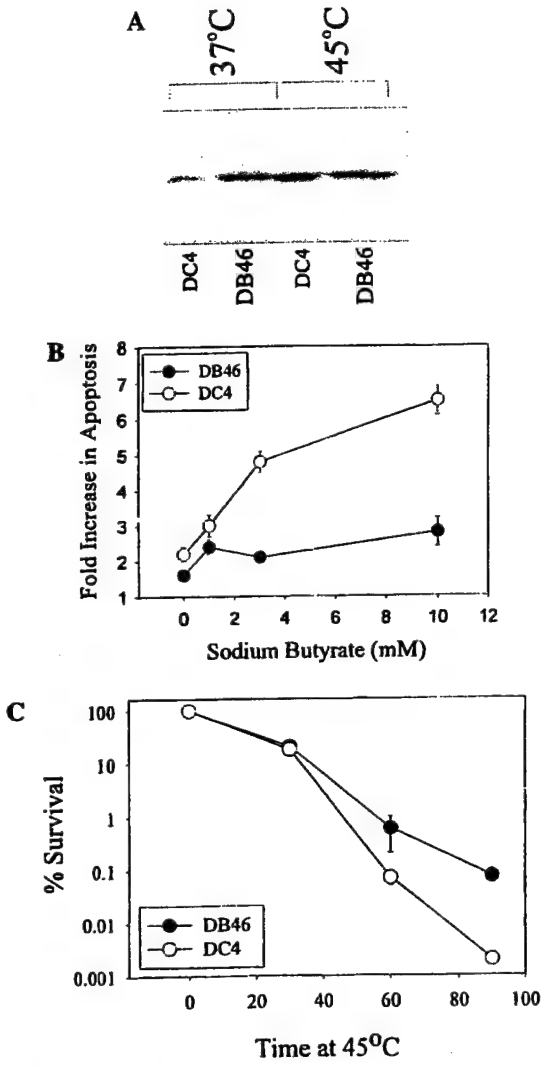

FIG. 1. DB46 cells overexpress hsp27. (A) Western blot for hsp27 in resting (37°C) and heat shocked (45°C) DB46 cells transfected with hsp27. As a control, parallel analysis was performed on lysates from DC4 cells transfected with a control plasmid. Note that at both 37°C and 45°C, DB46 cells contain approximately two- to three-fold more hsp27 than control cells. (B) Dose response analysis of sodium butyrate-induced apoptosis. Note that DB46 cells exhibit four-fold less apoptosis as measured by ELISA than control cells in response to 10 mM sodium butyrate. (C) Dose response analysis of % survival of colonies following heat shock at 45°C.

hsp27 Overexpression Does Not Alter Binding of DB46 Cells to Laminin-5

Because laminin-5 plays a significant role in controlling breast cell growth, migration, and differentiation (8, 9), we compared adhesion of DB46 and DC4 cells to laminin-5 using 30 minute cell adhesion assays. We found no difference between cell lines in adhesion to purified laminin-5 (Fig. 2). Laminin-5 was one of the most preferred adhesive substrates compared to additional ECM proteins (laminin-1, collagen IV, fibronectin).

Phosphorylation of hsp27 by MAP Kinase Enhances Migration of DB46 Cells on Laminin-5

We determined the impact of hsp27 over-expression on haptotactic migration towards laminin-5 using *in vitro* Transwell assays. DB46 cells retained the constitutive migration of parental MDA-MB-231 cells on laminin-5, as did control DC4 cells (Fig. 3). Therefore, hsp27 overexpression alone does not significantly affect migration of DB46 cells. Under these conditions, hsp27 was weakly phosphorylated in both DB46 and DC4 cells. However, stimulation of hsp27 by exposure to 41°C, or via activation of MAP kinase with 10 ng/ml anisomycin, led to a significant (29–53%) enhancement of migration in DB46 cells (Fig. 3A). Under these conditions, both ERK 1/2 and hsp27 were phosphorylated, as determined by western blot with anti-phospho-ERK 1/2 antibody (Fig. 3B) and immunoprecipitation of hsp27 from $^{32}PO_4$ radiolabeled cells (Fig. 3C). Addition of the MEK1 (upstream activator of ERK 1/2) inhibitor PD98059 did not significantly inhibit migration under normal conditions. These results indicate that hsp27 phosphorylation by heat or MAP kinase activation enhances migration. While hsp27 phosphorylation correlated with enhanced migration in DB46 cells, the degree of migration enhancement varied with the relative phosphorylation of MAP kinase.

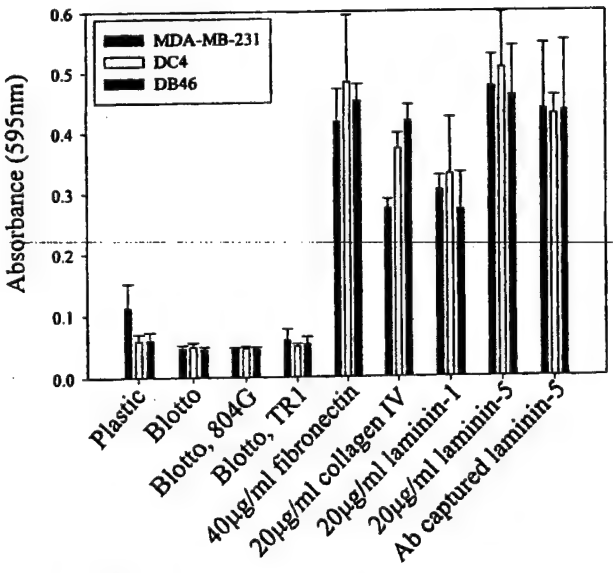

FIG. 2. hsp27 overexpression does not alter cell adhesion of DB46 cells to laminin-5. DB46, DC4, and parental MDA-MB-231 cells were plated on the indicated ECM proteins for 30 minutes, gently washed to remove unbound cells, then fixed, stained, and quantitated as previously described using mouse laminins, collagen IV and bovine fibronectin. TR1 = mouse monoclonal anti-laminin-5 antibody, 804G = conditioned medium from rat 804G cells containing laminin-5. Laminin-5 was captured by affinity method using TR1 antibody as described previously. Data are presented as statistical mean ± standard deviation (n = 16).

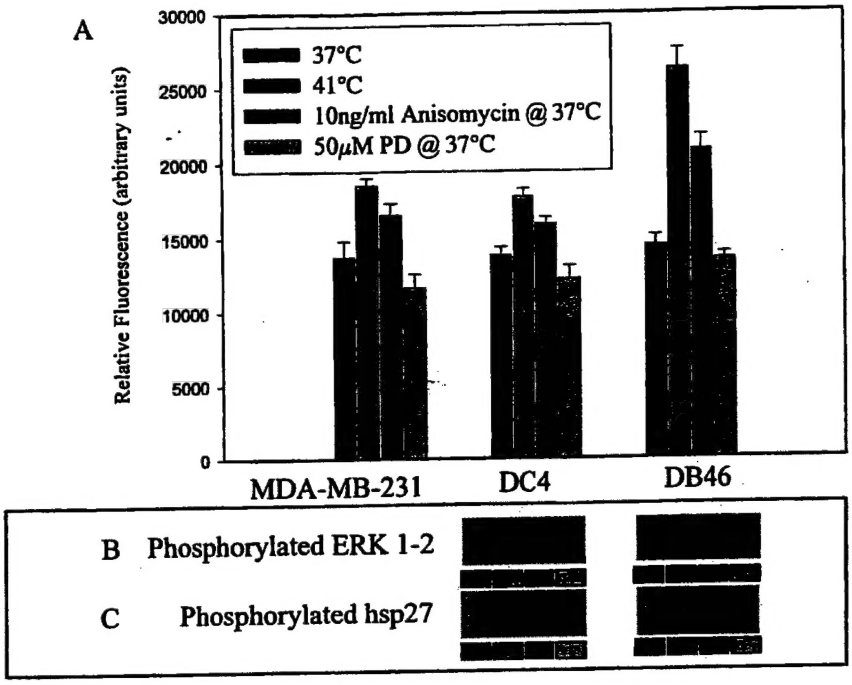

FIG. 3. Phosphorylation of hsp27 by MAP kinase enhances migration of DB46 cells on laminin-5. (A) Haptotactic migration assay on laminin-5. During the entire migration period, cells were exposed to mild heat shock (41°C), 10 nM anisomycin, or 50 μM PD98059. Results expressed as the statistical mean of the mean measurement from each filter, ± standard error of the means (n = 4). (B) Western blot analysis of ERK 1/2 phosphorylation with anti phospho-MAP kinase antibody. Cells were exposed to the indicated conditions for 30 minutes, then lysed and processed for western blot analysis. (C) Immunoprecipitation analysis of phosphorylated hsp27.

DB46 Cells Resist a Subset of Migration Inhibiting Drugs

hsp27 overexpression confers resistance to drugs that induce apoptosis and inhibit growth, possibly by modulating intracellular signaling pathways. To test the hypothesis that hsp27 influences laminin-5-associated migration signaling pathways, we repeated our cell migration assays in the presence of several drugs that inhibited intracellular signaling molecules and reduced migration of parental cells.

We found that DB46 cells were less sensitive to a subset of these drugs than the DC4 control cells. Specifically, these cells were less sensitive to the migration inhibiting effects of pertussis toxin (1 μg/ml) (74% vs. 51% of control migration), which inhibits activation of the heterotrimeric G protein subunit $G\alpha_i$; and completely insensitive to a sublethal dose of a specific inhibitor of PKG (KT5823, 10 μg/ml) (96% vs. 43% of control migration) (Fig. 4). In contrast DB46 cells were equally sensitive to inhibitors of adenylate cyclase (SQ22536, 50 μM), protein kinase A (H89, 10 μM), protein kinase C (bisindolylmaleimide, 10 μM) and protein tyrosine kinases (genistein, 50 μM) (Fig. 4). Thus, hsp27 protection under these conditions is not broadly applied but specific to certain signaling pathways involving at least PKG and $G\alpha_i$.

The hsp27 That Confers Resistance to Migration-Inhibiting Drugs Is Unphosphorylated

Because hsp27-enhanced migration of DB46 cells is phosphorylation-dependent, we determined the phosphorylation state of hsp27 under conditions where it

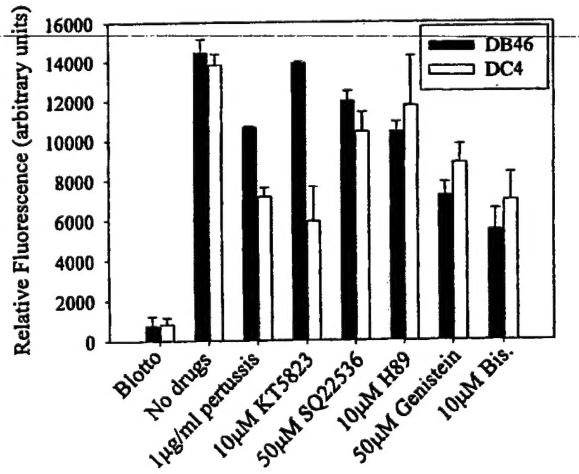

FIG. 4. DB46 cells resist a subset of migration-inhibiting drugs. Migration assays were performed as in Fig. 3, except that cells were plated in the presence of indicated concentrations of inhibitory drugs. Results expressed as the statistical mean of the mean measurement from each filter, ± standard deviation (n = 4).

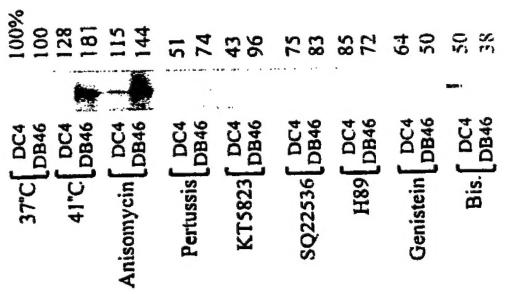

FIG. 5. Correlation of migration to hsp27 phosphorylation state. Percent migration data (above) obtained from Figs. 3A and 4. Immunoprecipitation analysis of phosphorylated hsp27 performed as described in Materials and Methods using anti hsp27 Ab.

conferred resistance to migration inhibiting drugs. Identical concentrations of drugs used to inhibit migration were administered to $^{32}PO_4$-labeled cells for 30 minutes, and hsp27 phosphorylation was determined by immunoprecipitation and autoradiography. Except for modest phosphorylation by bisindolylmaleimide, no drug treatment induced hsp27 phosphorylation over background levels (Fig. 5). These results demonstrate that hsp27 plays two discrete roles in controlling migration in DB46 cells: unphosphorylated hsp27 protects against the migration-inhibiting effects of pertussis toxin and KT5823, while only phosphorylated hsp27 enhances MAP kinase-dependent migration. Percent migration relative to control (37°C, no drugs) is indicated above band for each treatment.

Enhancement of Migration by Phosphorylated hsp27 Correlates with Its Relocalization to the Nucleus

Phosphorylation of hsp27 can affect its intracellular localization (18). Because we observed that hsp27 phosphorylation distinguished between its migration-enhancing or drug resisting effects, we determined its subcellular localization under both conditions. Indirect immunofluorescence microscopy revealed that, in the absence of heat shock or drug treatments, hsp27 was distributed throughout the cytosol with a slight concentration in the perinuclear region (Fig. 6, panel B). Upon heat stimulation or treatment with anisomycin, hsp27 relocated to the nucleus within 45 minutes (Fig. 6, panels F, H). In contrast, hsp27 did not relocalize in cells treated with pertussis toxin (Fig. 6, panel D).

From the above data, we conclude that hsp27 plays two distinct roles in controlling migration of DB46 cells on laminin-5: when phosphorylated, hsp27 concentrates in the nucleus and enhances constitutive migration, while unphosphorylated hsp27 remains distributed throughout the cytosol and offers resistance to a subset of signaling inhibitors that reduce constitutive migration.

DISCUSSION

Our data present three lines of evidence that hsp27 acts in two distinct roles to affect cell migration. First, activation of MAP kinase cascades by anisomycin leads to phosphorylation of hsp27 and enhanced migration. While overexpression of hsp27 can enhance cell migration in a phosphorylation dependent manner (19), the upstream signaling mechanisms responsible for mediating this effect are not well defined. In endothelial cells, p38 and SAPK2, but not ERK 1/2, mediate hsp27-dependent modulation of the actin cytoskeleton and chemotactic migration (4, 20, 21). However, the MAP kinase ERK 1/2 is implicated in ECM mediated events and may be involved in haptotactic migration on laminin-5 (22). Inhibition of ERK 1/2 by PD98059 did not inhibit haptotactic migration on laminin-5, though it's phosphorylation by anisomycin did correlate with enhanced migration. This result does not rule out the involvement of p38 and SAPK, both of which are activated by anisomycin (23).

Second, unphosphorylated hsp27 offers protection against inhibitors of specific intracellular signaling molecules in our cells. Hsp27 selectively protects against cytotoxic compounds, some of which stimulate intracellular signaling cascades (24, 25), perhaps by acting as a molecular chaperone (24); this protection is not always phosphorylation dependent (26). In our cells, hsp27 confers resistance to the repression of migration by inhibitors of PKG and adenylate cyclase, but not inhibitors of PKA and PKC. These results raise the possibility that hsp27 may function as a molecular chaperone or signaling molecule to maintain activity of a subset of signaling pathways required for constitutive migration of malignant tumor cells.

Third, hsp27 redistributes to the nucleus and sites of actin polymerization when phosphorylated, and remains cytosolic when unphosphorylated. This differential distribution correlates with the two distinct functions we observe in our cells, enhancement of migration, and protection against signaling inhibitors. Others have reported similar changes in distribution of hsp27 in response to serum stimulation (2, 18), although re-localization in response to heat shock may be independent of hsp27 phosphorylation (5).

In summary, the most significant finding of this study is that hsp27 affects migration of breast cancer cells by two distinct mechanisms. Further, these mechanisms can be distinguished on the basis of hsp27 phosphorylation state and intracellular localization. Because hsp27 is thought to act as a molecular chaperone, our data support a model whereby unphosphorylated hsp27 sequesters damaged signaling molecules, thereby conferring resistance to drugs that otherwise inhibit haptotactic migration of breast cancer cells. Further, because phosphorylated hsp27 functions as an actin capping protein *in vitro* (4), this may explain why it enhances constitutive

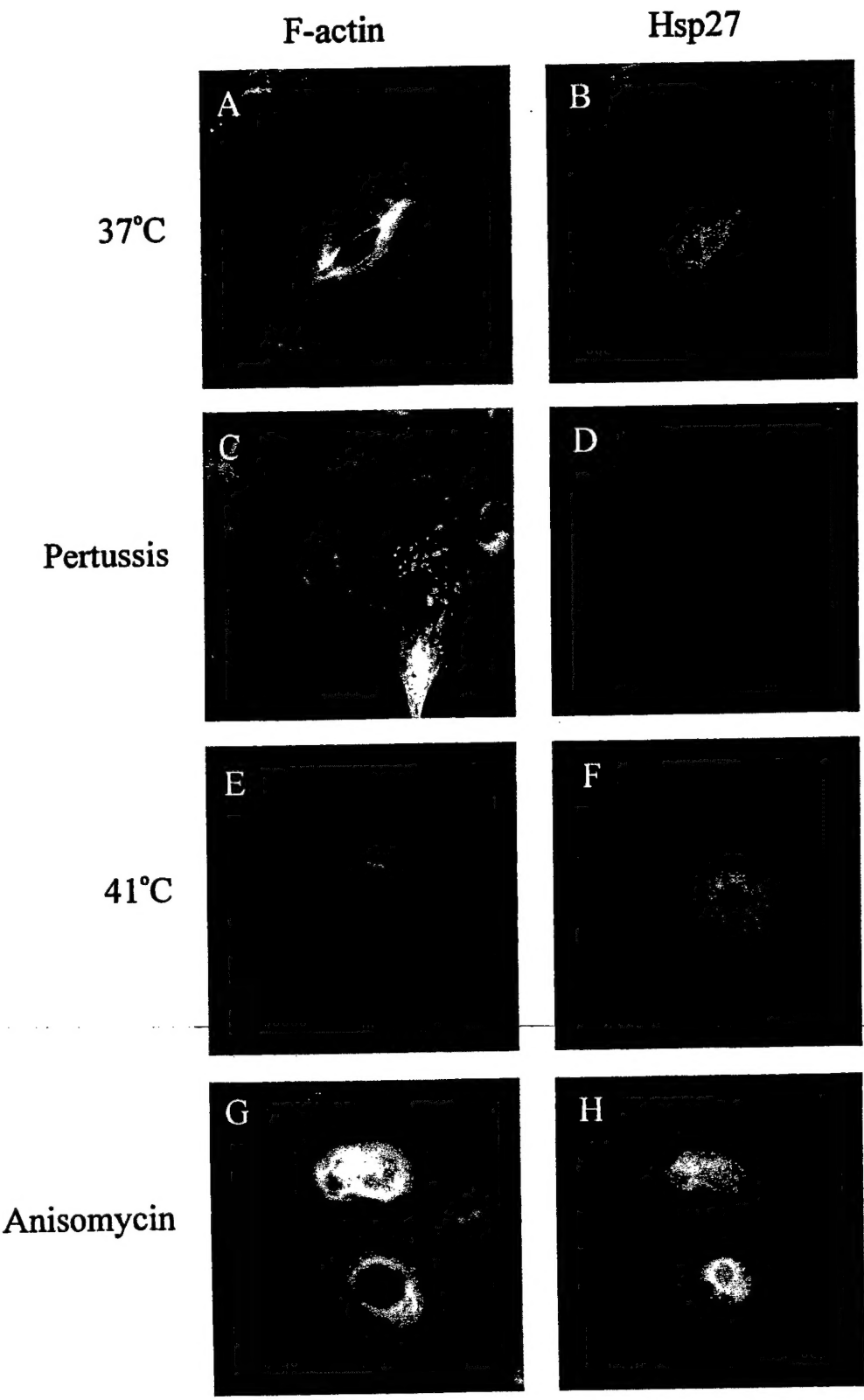

FIG. 6. Localization of hsp27 and f-actin. Fluorescence micrographs of DB46 cells doubly stained for actin (phalloidin) and hsp27. Magnification = 400×.

migration of breast cancer cells: In our model, phosphorylation of hsp27, coupled with a haptotactic migration stimulus (e.g., binding to ECM proteins such as laminin-5), may enhance actin filament assembly within the leading edge of migrating cells.

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